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1. APPL.ENVIRON.MICROBIOL 1998, 64;2247-2255

2. APPL.ENVIRON.MICROBIOL 1999 65; 3710-3713

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Streptococcus gordonii Biofilm Formation: Identification of Genes that Code for Biofilm Phenotypes

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Viridans streptococci, which include *Streptococcus gordonii*, are pioneer oral bacteria that initiate dental plaque formation. Sessile bacteria in a biofilm exhibit a mode of growth that is distinct from that of planktonic bacteria. Biofilm formation of *S. gordonii* Challis was characterized using an in vitro biofilm formation assay on polystyrene surfaces. The same assay was used as a nonbiased method to screen isogenic mutants generated by Tn*916* transposon mutagenesis for defective biofilm formation. Biofilms formed optimally when bacteria were grown in a minimal medium under anaerobic conditions. Biofilm formation was affected by changes in pH, osmolarity, and carbohydrate content of the growth media. Eighteen biofilm-defective mutants of *S. gordonii* Challis were identified based on Southern hybridization with a Tn*916*-based probe and DNA sequences of the Tn*916*-flanking regions. Molecular analyses of these mutants showed that some of the genes required for biofilm formation are involved in signal transduction, peptidoglycan biosynthesis, and adhesion. These characteristics are associated with quorum sensing, osmoadaptation, and adhesion functions in oral streptococci. Only nine of the biofilm-defective mutants had defects in genes of known function, suggesting that novel aspects of bacterial physiology may play a part in biofilm formation. Further identification and characterization of biofilm-associated genes will provide insight into the molecular mechanisms of biofilm formation of oral streptococci.

Viridans streptococci, which include *Streptococcus gordonii*, are pioneer bacteria that initiate the formation of biofilms on tooth surfaces known as dental plaque. These ubiquitous initial colonizers constitute a majority of the cultivable bacteria found in dental plaque (46) and are the most frequent etiologic agents of bacterial endocarditis (16, 61). Over the last decade, viridans streptococci have become significant opportunistic pathogens and a major cause of bacteremia in immunocompromised patients, accounting for 40% of infections in neutropenic patients (26, 33).

Dental plaque forms by two distinct sequential steps: adhesion of early colonizers to host tissue components (24, 53) and then time-dependent accumulation of multi-layered cell clusters embedded in a matrix of bacterial and host constituents (4, 55, 62). Although numerous studies have elucidated the mechanisms of initial streptococcal adhesion (7, 13, 17, 22, 25, 31, 51, 52), the subsequent process of bacterial accumulation and proliferation leading to functionally heterogeneously organized sessile communities called dental plaque is poorly understood.

Genetic and molecular studies of dental plaque have predominantly used planktonic bacteria grown in batch culture. Although these studies have provided extensive and relevant information, dental plaque bacteria colonize and exist as sessile bacterial communities. Biofilm formation is initiated by interactions between planktonic bacteria and a surface in response to appropriate environmental signals. A fully developed, surface-attached dental biofilm is highly structured, with distinct architectural and physiochemical properties commonly observed with other biofilm communities (10, 11). Biofilm bacteria exhibit a distinct mode of growth which differs from that of planktonic cells and which is characterized by an increased resistance to antibiotics and differences in levels of gene expression and cellular physiology (10, 11, 29). Therefore, we hypothesize that novel, biofilm-associated genes are required for the development of dental biofilms after initial bacterium-surface contact is established. As sessile populations reflect conditions in vivo more accurately than planktonic bacteria, the genes expressed by biofilm bacteria are likely to play a role in the colonization of tooth surfaces and the increased virulence exhibited by viridans streptococci in susceptible hosts (16, 26, 33, 61). The aims of this study were to determine the influence of various environmental factors on the in vitro biofilm formation of *S. gordonii* Challis and to identify genes involved in the biofilm formation of this species of viridans streptococci.

The utility of polystyrene as a surface for attachment by marine pseudomonads under various physiological conditions was demonstrated by Fletcher (20). Microtiter plates made of polystyrene provide a convenient and sterile abiotic surface for studying bacterial biofilm formation. Studies using abiotic surfaces coupled with transposon mutagenesis have identified novel genes in the biofilm formation of *Staphylococcus epidermidis* (28, 43, 56), *Escherichia coli* (50), and *Pseudomonas fluorescens* (47, 48). Since tooth surfaces, generally covered by a thin pellicle layer of salivary origin, are abiotic, a similar strategy with Tn*916* transposon mutagenesis was used in this study as a nonbiased method of isolating biofilm-defective mutants of *S. gordonii* Challis and identifying genetic loci that are associated with biofilm formation.

MATERIALS AND METHODS

Bacterial strains, media, and chemicals. *S. gordonii* Challis 2, a rifamycin-resistant (500 μg/ml) strain of *S. gordonii* Challis (42), was used as the parent strain. Other oral streptococcal strains used were obtained from the American Type Culture Collection, except for *Streptococcus parasanguis* FW213 (from P. Fives-Taylor, University of Vermont) and *Streptococcus oralis* C104 (from P. Kolenbrander, National Institute of Dental and Craniofacial Research). All strains were grown in Todd-Hewitt broth with 0.2% yeast extract (THB+YE). Antibiotics were added at the following concentrations: for *E. coli*, 100 μg of ampicillin ml^{-1} and 4 μg of tetracycline ml^{-1}, and for *S. gordonii*, 10 μg of tetracycline ml^{-1}. All enzymes for DNA manipulations were purchased from Promega (Madison, Wis.).

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Biofilm assay. The biofilm formation assay used is based on the method by O'Toole and Kolter (48). A minimal, defined medium modified from the work of Carlsson (6) and Jenkinson (30) was used as the biofilm medium (BM). The BM contained 58 mM K_2HPO_4, 15 mM KH_2PO_4, 10 mM $(NH_4)_2SO_4$, 35 mM NaCl, 0.8% (wt/vol) glucose, 0.2% (wt/vol) Casamino Acids (CAA), and 100 mM $MnCl_2 \cdot 4H_2O$ (pH 7.4) and was supplemented with filter-sterilized vitamins (0.04 mM nicotinic acid, 0.1 mM pyridoxine HCl, 0.01 mM pantothenic acid, 1 μM riboflavin, 0.3 μM thiamin HCl, and 0.05 μM D-biotin), amino acids (4 mM L-glutamic acid, 1 mM L-arginine HCl, 1.3 mM L-cysteine HCl, and 0.1 mM L-tryptophan), and 2 mM $MgSO_2 \cdot 7H_2O$.

Round-bottomed microtiter plates (Becton Dickinson Labware, Lincoln Park, N.J.) containing 100 μl of BM per well were inoculated with *S. gordonii* Challis (4.2×10^6 CFU per well) from a 16-h growth in THB+YE without agitation. After 16 h of incubation at 37°C, 25 μl of 1% (wt/vol) crystal violet (CV) solution was added to each well. After 15 min, wells were rinsed three times with 200 μl of distilled H_2O and air dried. Bacterial growth and biofilm formation were quantified by measuring the absorbance at 575 nm (A_{575}) of the bacterial culture and CV-stained biofilm, respectively. Each assay was performed in triplicate. Biofilm formation was examined on both polystyrene (Falcon 3918) and polyvinylchloride (Falcon 3911) microtiter plates under aerobic and anaerobic conditions to determine the optimum conditions for the assay.

All the biofilm assays described below were performed on polystyrene plates under anaerobic conditions. Biofilm formation of *S. gordonii* Challis was examined in each of the following media: BM, BM without glucose, BM without CAA, THB, THB+YE, and Trypticase soy broth (TSB).

Bacterial growth and biofilm formation of *S. gordonii* Challis were assayed over 16 h to determine the relationship between growth and biofilm formation. The effects of changes in osmolarity on *S. gordonii* Challis biofilm formation were assessed by supplementing BM with the osmolyte NaCl (0 to 0.4 M). In addition, the influence of changes in pH on biofilm formation was examined by using BM with a starting pH that ranged from 3 to 12. The effects of replacing the glucose in BM with an alternative carbohydrate at a final concentration of 0.8% (wt/vol) were examined. The carbohydrates tested were arabinose, fructose, fucose, galactose, lactose, maltose, maltotriose, mannose, raffinose, ribose, rhamnose, sucrose, and xylose.

Biofilm formation of *S. gordonii* Challis and 15 other oral streptococci were assayed and categorized based on the A_{575} of the CV-stained biofilm. When the A_{575} of the CV-stained biofilm was greater than 2.0, the strain was categorized as a good biofilm former. Strains which produced CV-stained biofilms with an A_{575} of 1.0 to 2.0 were designated moderate biofilm formers, while those with CV-stained biofilms that had an A_{575} lower than 1.0 were categorized as poor biofilm formers.

SEM. Biofilm formation of *S. gordonii* Challis on polystyrene surfaces coated with three different matrix proteins was assayed. Laminin (2 μg/cm²), type IV collagen (10 μg/cm²), and fibronectin (5 μg/cm²) were each used to coat polystyrene microtiter plates according to the instructions of the manufacturer (Sigma-Aldrich Co., St. Louis, Mo.). Plates were air dried under UV and used in the biofilm assay as described previously. Biofilms that formed were examined by scanning electron microscopy (SEM) to verify the quantitative results observed. The microtiter plates were fixed by adding an equal volume of formaldehyde-glutaraldehyde to the medium for 1 h, followed by addition of a full-strength fixative overnight. The wells were washed three times with 0.1 N cacodylate buffer, postfixed with 2% OsO_4 in *s*-collidine buffer for 2 h, and rinsed with water. Following dehydration through a graded series of ethanol, the collars of the wells were cut away with a band saw. The wells were rinsed with ethanol and air dried. The 96-well plate was then cut into six equal parts, and the upper left corner of each section was marked for orientation of the wells. The sections were mounted on aluminum stubs, coated with palladium-gold, and examined in a JEOL 6400 scanning electron microscope. The sections were tilted 45° in order to examine the sides of wells. Wells for photography were randomly selected, but the area within the well for viewing was always measured to be 2 mm from the cut edge of the collar, thus avoiding the bacteria which may have settled at the bottom of the well during incubation. Polaroid photographs were taken of representative areas and digitized with a scanner (Agfa Corp., Ridgefield, N.J.) for reproduction.

Tn*916* transposon mutagenesis. *S. gordonii* Challis grown in THB+YE was diluted (1:100) in streptococcal transformation medium (39). The medium consisted of 1% Proteose Peptone, 0.2% YE, and 0.2% glucose (pH 7.8) and was supplemented with 10% heat-inactivated horse serum (Sigma). After inoculation, the culture was incubated anaerobically at 37°C for 105 min for maximum transformation efficiency (39). After incubation, 2 to 3 μg of pAM120 (pGL101 containing pAD1*EcoRI*F::Tn*916*) plasmid DNA was mixed with 0.5 ml of the culture and the mixture was incubated for another 4 h under anaerobic conditions at 37°C. Cells were then plated on brain heart infusion agar (BBL Microbiology Systems, Cockeysville, Md.) containing tetracycline and incubated anaerobically for 48 h. The plasmid DNA for transformation was isolated from *E. coli* CG120 (23) using a maxi prep kit (Qiagen Inc., Valencia, Calif.). The transformation frequency was 3×10^3 transformants per μg of DNA. Each tetracycline-resistant colony was picked and transferred into a well on a microtiter plate containing 200 μl of BM with tetracycline. After the inoculum was mixed, 100 μl of the BM was transferred to another well on a new microtiter plate to create a duplicate. Both plates were then incubated anaerobically at 37°C for 24 to 48 h. After incubation, bacterial growth in each well was determined by recording the A_{575} of the culture, and one plate was stained with 1% CV. Bacteria from the duplicate plate corresponding to wells with equivalent levels of growth but poor CV staining were picked as putative biofilm formation-defective mutants. These putative mutants were streaked onto brain heart infusion agar containing tetracycline and retested for their ability to form biofilms to rule out false positives. Colonies that continued to show defective biofilm formation were designated biofilm-defective mutants.

Molecular techniques. Chromosomal DNA was isolated from the wild-type *S. gordonii* Challis and putative biofilm-defective mutants by the method described by Ganeshkumar et al. (22). DNA was digested with *Hin*dIII, separated by agarose gel electrophoresis, and transferred onto a nitrocellulose membrane for Southern hybridization. The probe used for hybridization was pAM120 labeled with digoxigenin (DIG) using the DIG DNA labeling system, according to the instructions of the manufacturer (Roche Molecular Biochemicals, Indianapolis, Ind.). After hybridization, the membrane was developed by enzyme immunoassay using a DIG nucleic acid detection kit (Roche Molecular Biochemicals).

DNA sequences flanking the transposon were determined by inverse PCR and DNA sequencing. Chromosomal DNAs from the mutants were digested with *Hin*dIII, self-ligated with T4 DNA ligase, and used as the PCR templates. Primers with *Eco*RV adapters (underlined) were designed using the published Tn*916* sequence (19). Primer IPTNL3 (5′ CGGATATCCGTAAAGTATCCG GAGAATA) was from positions 11990 to 12015 of the Tn*916* coding strand, about 180 nucleotides upstream of a unique *Hin*dIII site, while primer IPTNR2 (5′ CGGATATCCGTTTGAAGTGTCTACCTAT) was from positions 191 to 174 of the Tn*916* antisense strand. PCR was carried out as follows. After initial denaturation for 2 min at 95°C, 36 cycles of amplification consisting of denaturation for 45 s at 94°C, annealing for 45 s at 53°C, and extension for 2 min at 72°C were performed. This procedure was followed by a final extension of 10 min at 72°C. The PCR products were analyzed by agarose gel electrophoresis, purified using a Qiagen PCR purification kit, and sequenced at the Molecular Genetics Core Sequencing Facility at The Forsyth Institute using a model 377 automated sequencer (Applied Biosystems, Foster City, Calif.). Each sequence obtained was compared with those in GenBank by using the BLASTX program (1).

Nucleotide sequence accession numbers. The nucleotide sequences of the sites of Tn*916* insertion in the different biofilm mutants have been deposited in GenBank under accession numbers AF207576 to AF207592 as indicated in Table 1.

RESULTS

***S. gordonii* biofilm formation is nutrient dependent.** Motile, gram-negative bacteria have been used previously to study biofilm formation on abiotic surfaces (20, 47, 48, 50). In this study, the biofilm formation of a nonmotile gram-positive oral bacterium, *S. gordonii* Challis, was examined using an assay based on the method described in a previous study (48). In this assay, staining with 1% CV for 15 min enables the visualization of attached, sessile cells after bacterial biofilms have formed in microtiter plate wells for 16 h. Unattached, planktonic cells are removed by rinsing with water. Cells are stained purple with CV (A_{575} of 1 is equivalent to approximately 2×10^6 CFU/well), whereas abiotic surfaces are not stained. As this assay lasts only over 16 h, it is biased towards initial events, which might not allow identification of defects in late biofilm formation.

Biofilm assays were carried out under various conditions to determine the optimum experimental conditions. Biofilms were readily apparent on polystyrene but not polyvinylchloride surfaces, and both growth and biofilm formation were greater under anaerobic conditions (Fig. 1). Anaerobically grown cells were found to form heavier, more uniform biofilms on both surfaces than aerobically grown cells. As the bacteria were grown without agitation, the cells that did not form a biofilm settled to the bottom of U-shaped wells and were effectively removed by washing. The facultative anaerobe *S. gordonii* formed biofilms on the lower part of the wells on the U-shaped surfaces below the air-liquid interface, in contrast to aerobes such as *P. aeruginosa* and *E. coli*, which grow at the air-liquid interface (48, 50).

When different media were used in the biofilm assay, only BM produced uniform biofilms (Fig. 1). No growth occurred when glucose or CAA was omitted from BM. In enriched

TABLE 1. Genetic identification[a] of biofilm-defective mutants of *S. gordonii* Challis

Mutant	Allele	Locus	% of level of wild-type biofilm formation[b]	Genetic function	Putative role in biofilm	Nucleotide sequence at Tn*916* junction[c]	Accession no.	Reference
1	8F9	*comD*	61.4	Histidine kinase	Quorum sensing	TTAATTTTATTGTAA	U80077	42
2	1C1	PBP 2B gene	9.0	Peptidoglycan biosynthesis	Osmoregulation?	AAGATTTTTATACTA	AF207585	This study
3	11E5L	PBP 5 gene	44.3	Peptidoglycan biosynthesis	Osmoregulation?	ATTATTTTTTAAAGA	AF207578	This study
	11E5U	ATPase gene		Cation transport	Osmoregulation?	CTATATTTTTAAAGA	AF207579	This study
4	11B4	*glmM*	55.5	Peptidoglycan biosynthesis	Osmoregulation?	GTTATTTTTTTATTA	AF207577	This study
5	15B3	*bacA*	12.6	Peptidoglycan biosynthesis	Osmoregulation?	TACTTTTATACTTTA	AF207587	This study
6	9F8	*abpA*	16.2	α-Amylase binding	Adhesion	CTCCTTTTTTATGTA	AF035817	51
7	29E5	*appC*	24.9	Oligopeptide transport	Nutrient sensing?	AGTTTTTTTTATGTA	AF207584	This study
8	4B3	*mutT*	17.1	DNA replication	Unknown	TTTATTATTAATTTA	AF207581	This study
9	13A12	*ytmP*	14.6	Unknown	Unknown	TTATTTTTTTTATTA	AF207592	This study
10	8A11	No match	35.3	Unknown	Unknown	GTATTTTTTACAAAA	AF207586	This study
11	6C12	No match	28.2	Unknown	Unknown	ATAATTTTTTATTTA	AF207591	This study
12	14H6	No match	26.1	Unknown	Unknown	GTTCTTTTTATTCAA	AF207582	This study
13	8B8	No match	33.2	Unknown	Unknown	TGATTTTTTTGAATA	AF207583	This study
14	12E11	No match	18.5	Unknown	Unknown	CTATATTTTTAAAGA	AF207576	This study
15	8G2	No match	21.6	Unknown	Unknown	CGAATTTTAATTTTA	AF207580	This study
16	29F1	No match	43.3	Unknown	Unknown	GTTCTTTTTAATGTA	AF207590	This study
17	18D10	No match	22.1	Unknown	Unknown	GCTATTATTATACCA	AF207588	This study
18	11G1	No match	32.1	Unknown	Unknown	TCGATTTTTTTATTA	AF207589	This study

[a] Based on DNA homologies with DNA sequences in GenBank.
[b] Wild-type biofilm formation was considered to be 100%.
[c] Fifteen nucleotides upstream of the chromosomal DNA of the Tn*916* insertion.

media, such as THB, THB+YE, and TSB, bacteria grew as well as in BM but failed to attach to the surface evenly, instead forming pellets at the bottom of the well. It appears that a nutritionally rich environment does not favor *S. gordonii* biofilm formation on polystyrene but that a nutritionally limited environment increases sessile growth.

When bacterial growth and biofilm formation of *S. gordonii* Challis were assayed over 16 h, a typical exponential growth curve was observed whereas biofilm formation increased linearly up to 10 h, at which time the CV-stained biofilm plateaued (at an A_{575} of ~2.5). Although growth was minimal up to 6 h, biofilm formation increased steadily during this time (data not shown). Due to these preliminary observations, subsequent biofilm assays were performed on polystyrene microtiter plates containing BM (the defined, minimal medium containing glucose and CAA), which resulted in distinct biofilms after 16 h of anaerobic incubation at 37°C.

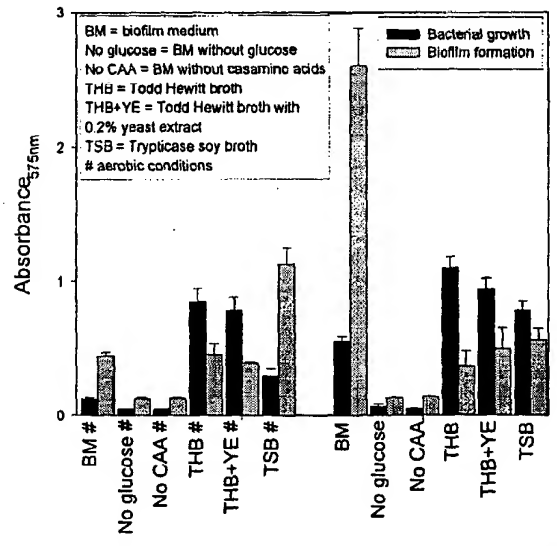

FIG. 1. Bacterial growth and biofilm formation of *S. gordonii* Challis under different growth conditions. Bacteria were grown in either BM, BM without glucose, BM without CAA, THB, THB+YE, or TSB. Growth and biofilm formation were measured under aerobic (#) and anaerobic conditions. All assays were performed in triplicate, and mean values and standard deviations are shown.

When the osmolarity of the BM was increased (Fig. 2), an initial increase in bacterial growth (at 0.1 to 0.3 M NaCl) was followed by a decrease in growth (at 0.4 M NaCl). In contrast, biofilm formation was reduced when osmolarity was increased from 0.1 to 0.4 M NaCl. When the starting pH of the BM was altered, biofilm formation was reduced at pH levels below 6 or above 8 while growth was reduced only when the pH was below 6 or above 10.5. Generally, biofilm formation was more sensitive to pH changes than bacterial growth. Maltose, mannose, sucrose, fructose, galactose, lactose, and maltotriose were found to be viable alternative carbon sources which could be used without adversely affecting growth or biofilm formation. The remaining carbohydrates examined, xylose, ribose, fucose, arabinose, raffinose, and rhamnose, did not support bacterial growth; therefore, no biofilm formation was seen.

Biofilm formation of *S. gordonii* Challis and 15 other oral streptococci were assayed and categorized based on the A_{575} of the CV-stained biofilm (Fig. 3). Four of the 16 strains tested, namely, *S. gordonii* Challis, *S. gordonii* 12396 and 33399, and *Streptococcus sanguis* 10556, were good biofilm formers. Three of the tested strains were moderate biofilm formers, consisting of *S. gordonii* 10558, *S. oralis* 11427, and *S. parasanguis* 15909, while 9 of the 16 strains (the largest group) were poor biofilm formers, consisting of *Streptococcus anginosus* 10713, *Streptococcus constellatus* 2226, *Streptococcus mitis* 4212 and 99456, *S. oralis* 10557 and C104, *S. parasanguis* FW213 and 15911, and *Streptococcus intermedius* 27335. The biofilm medium used was optimized for *S. gordonii*; hence, other oral streptococcal species such as *S. anginosus* 10713, *S. intermedius* 27335, *S. parasanguis* FW213, and *S. oralis* 11427 were found to grow poorly

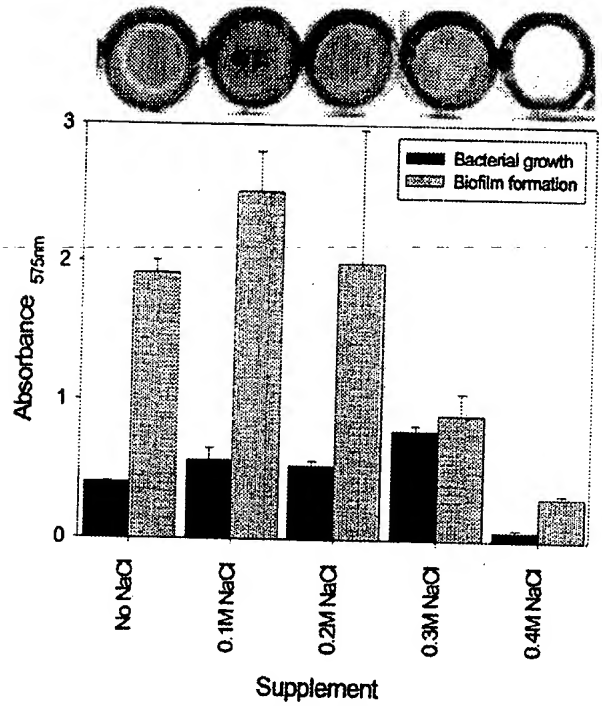

FIG. 2. Bacterial growth and biofilm formation of *S. gordonii* Challis in BM with different levels of osmolarity. The NaCl supplement in BM varied from 0 to 0.4 M. Assays were performed using BM and polystyrene plates under anaerobic conditions. A representative row of CV-stained microtiter plate wells is shown above the graph.

in this medium. At the same time, *S. oralis* C104 grew very well in BM but was unable to form biofilm on polystyrene surfaces. These results indicate a wide variation in the abilities of oral streptococci grown in BM to form biofilms on polystyrene. Variation in the ability to form biofilms was also observed within the same species.

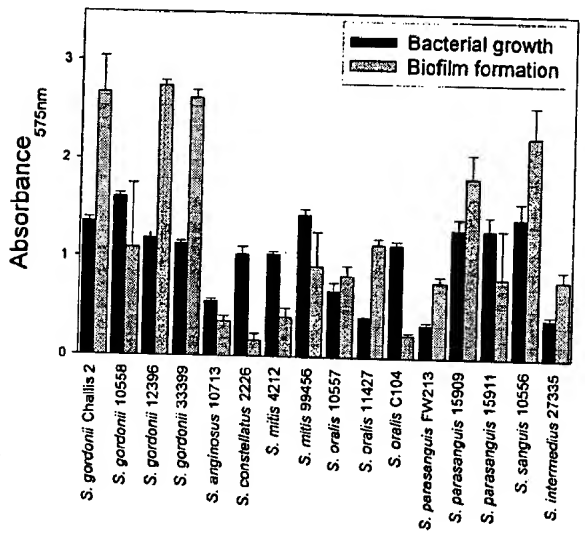

FIG. 3. Bacterial growth and biofilm formation of various oral streptococci. Assays were performed using BM and polystyrene plates under anaerobic conditions.

In order to determine whether mammalian matrix proteins affect the biofilm formation of *S. gordonii* Challis, polystyrene surfaces coated with laminin, type IV collagen, and fibronectin were used. Polystyrene plates coated with laminin and fibronectin had a slight reduction in biofilm formation, while uncoated polystyrene and the collagen-coated polystyrene exhibited no difference in biofilm formation (data not shown). As the results from both the quantitative assay and SEM (Fig. 4) demonstrate that biofilm formation on coated surfaces did not differ significantly from that on uncoated polystyrene, the latter could be justifiably used to screen for biofilm phenotypes. Interestingly, coated surfaces showed some structural differences, with more cell clusters, whereas uncoated polystyrene showed a uniform distribution of biofilm bacteria. The use of surfaces coated with matrix proteins can facilitate the isolation of mutants that are defective in binding to proteins on damaged heart valves and subsequent identification of bacterium-host interactions that are important in biofilm formation and the pathogenesis of endocarditis (59, 60).

Screening for *S. gordonii* biofilm-defective mutants. A total of 18 (0.07%) biofilm-defective mutants were isolated after ·25,000 transposon mutants generated by Tn*916* mutagenesis were screened using the biofilm assay described in this study (Fig. 5). Only mutants exhibiting an amount of growth similar to that of wild-type Challis were chosen for further study, as a mutation that confers a nonspecific growth defect can affect biofilm formation indirectly. The reduction in biofilm formation ranged from 39% (the 8F9 mutant) to 91% (the 1C1 mutant), with a majority (14 of 18) of the mutants having more than 60% reduction in biofilm formation (Table 1). Southern hybridization of *Hin*dIII-digested chromosomal DNA from each mutant with DIG-labeled pAM120 confirmed the presence of Tn*916*. As Tn*916* has a single *Hin*dIII site, the presence of two hybridizing bands is consistent with the mutant having only a single transposon insertion. All the mutants isolated had single insertions except for three with the mutations 11E5, 9F8, and 29F1, which had three hybridizing bands, indicating the presence of two transposon insertions. Hybridization results from nine of the biofilm-defective mutants, which will be discussed in further detail, are shown in Figure 6.

The DNA sequence flanking each Tn*916* insertion was determined in order to identify the gene(s) disrupted in each mutant. The number of nucleotides sequenced from the various mutants ranged from 53 to 886. Each sequence obtained was analyzed using the BLASTX program (1), which translates the DNA sequences in all six reading frames and compares these predicted protein sequences with those in GenBank. Eighteen biofilm-defective mutants of *S. gordonii* Challis were identified based on Southern hybridization and analyses of sequences of the Tn*916*-flanking regions (Table 1). The number of PCR products obtained from the mutants confirmed the number of transposon insertions indicated by the Southern hybridization results.

Biofilm-defective mutant 1 (8F9) had an insertion within the *comD* gene (65 nucleotides into the open reading frame [ORF]) in the competence locus (*comCDE* operon) of *S. gordonii* Challis (42). ComD is a histidine kinase that can act as an environmental sensor, and its activity is regulated in response to specific stimuli.

Bacterial cell wall peptidoglycan is a covalently closed, net-like polymer in which glycan strands are linked by peptides. Its structure is determined by many biosynthetic and autolytic reactions. The DNA sequences of Tn*916*-flanking regions in biofilm-defective mutants 2 (1C1), 3 (11E5), 4 (11B4), and 5 (15B3) identified homologs in peptidoglycan biosynthesis genes, including genes that encode penicillin-binding proteins (PBPs),

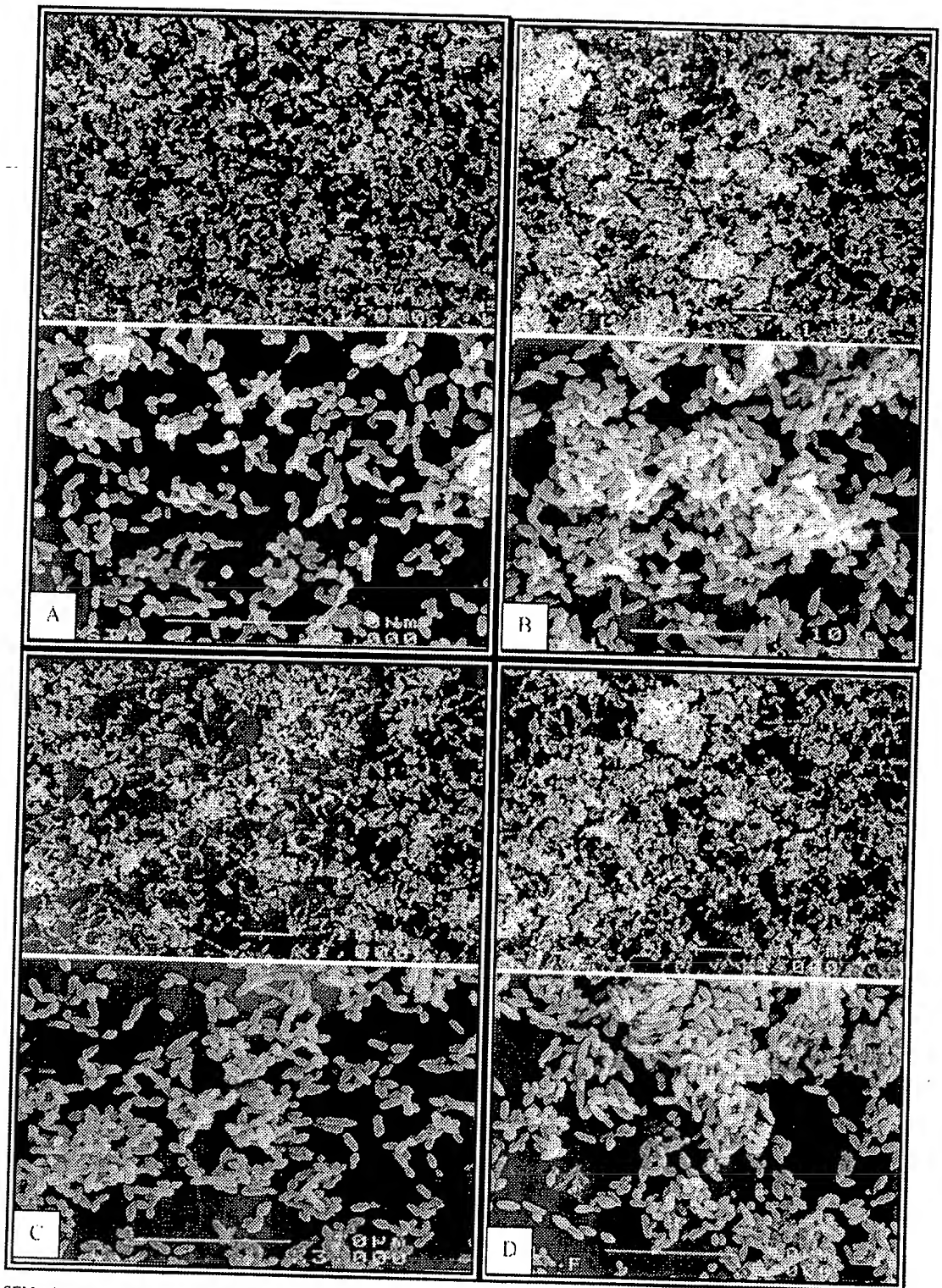

FIG. 4. SEM micrographs of *S. gordonii* Challis biofilm formation on uncoated and coated polystyrene surfaces. (A) Uncoated polystyrene; (B) laminin; (C) type IV collagen; (D) fibronectin. Magnification is shown by the bar (10 μm). Two different magnifications are shown for each surface (×1,000 for the upper images and ×3,000 for the lower images).

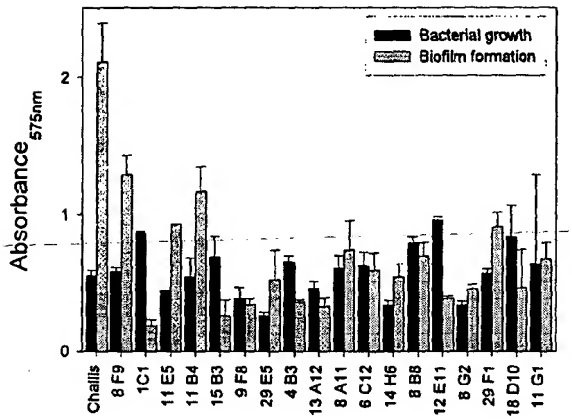

FIG. 5. Bacterial growth and biofilm formation of wild-type *S. gordonii* Challis and biofilm-defective mutants. Assays were performed using BM and polystyrene plates under anaerobic conditions.

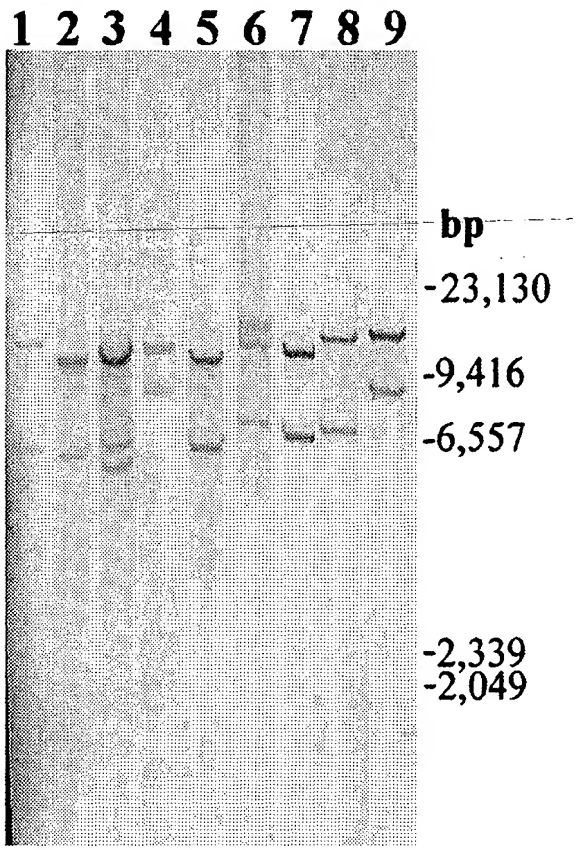

FIG. 6. Southern hybridization of *Hin*dIII-digested chromosomal DNAs from nine representative biofilm-defective mutants with a DIG-labeled pAM120 probe containing Tn*916*. Lanes: 1, 8F9 mutant; 2, 1C1 mutant; 3, 11E5 mutant; 4, 11B4 mutant; 5, 15B3 mutant; 6, 9F8 mutant; 7, 29E5 mutant; 8, 4B3 mutant; 9, 13A12 mutant. DNA sizes are shown on the right.

which are active-site serine transferases. Mutant 2 (1C1) has a transposon insertion in an ORF that is homologous to the gene encoding PBP 2B of *S. pneumoniae*, a transpeptidase from the class B high-molecular-weight PBPs.

Mutant 3 (11E5) had two transpositions that were separated after inverse PCR by agarose gel electrophoresis. The lower fragment, designated 11E5L, has a transposon insertion in an ORF homologous to the gene for PBP 5 of *Bacillus subtilis*, a DD-carboxypeptidase from the class A low-molecular-weight PBPs. The Tn*916*-flanking region in the higher fragment, designated 11E5U, has homology with a putative cation-transporting ATPase from *Mycobacterium tuberculosis*. This P-type (or E1 E2-type) ATPase belongs to a large family of prokaryotic and eukaryotic proteins that transport a variety of cations in various cellular processes. An insertion mutant of a P-type prokaryotic ATPase gene in *Synechococcus* sp. strain PCC7942 has demonstrated hypersensitivity to osmotic stress upon addition of NaCl or sorbitol to the medium (32), suggesting a possible role in osmoadaptation. Osmoadaptation is an adaptive response to changes in external turgor pressure and is mediated by a primary ATP-dependent (K^+-ATPase) transport system, since the accumulation of potassium ions is known to be the primary response to hypertonic stress in eubacteria. Either the 11E5L or the 11E5U transposition could be responsible for mutant 3 displaying the biofilm-defective phenotype.

The Tn*916*-flanking region of mutant 4 (11B4) has homology with *glmM*, a phosphoglucomutase gene in *E. coli*. GlmM catalyzes the formation of glucosamine-1-phosphate from glucosamine-6-phosphate, the first step in the biosynthetic pathway leading to the essential peptidoglycan precursor UDP-*N*-acetylglucosamine (44).

The Tn*916*-flanking region of mutant 5 (15B3) has homology with *bacA*, which encodes an undecaprenyl kinase involved in the lipid phosphorylation that confers bacitracin resistance in *E. coli*. The resistance is due to the tight binding of bacitracin to a complex of undecaprenyl diphosphate and a metal ion. This tight binding prevents undecaprenyl diphosphate from functioning as a membrane-associated carrier of intermediates during peptidoglycan biosynthesis (5).

Mutant 6 (9F8) had two transposon insertions, one of which is 23 nucleotides upstream of *abpA*, which encodes AbpA, an α-amylase-binding protein in *S. gordonii* Challis (51). In a previous study, the *abpA* gene was also identified by Tn*916* mutagenesis, with an insertion 15 nucleotides upstream of *abpA* (51). The second insertion in 9F8 was in a region with no homology in the DNA database. Either of these transpositions could be responsible for the biofilm-defective phenotype of the 9F8 mutant.

The Tn*916*-flanking region of mutant 7 (29E5) has homology with *appC*, which encodes a member of an oligopeptide (tetra- or pentapeptide) transport system in *B. subtilis*. This system may be a growth-monitoring communication system between cell wall synthesis systems and the cytoplasm which senses the turnover rate of cell wall peptides as a direct function of bacterial growth during sporulation (35). Similar mechanisms may be involved in modulating streptococcal growth during early dental plaque formation.

The Tn*916*-flanking region of mutant 8 (4B3) has homology with *mutT*, a member of the DNA replication machinery that prevents A · G mispairs. MutT is a nucleoside triphosphatase which prevents A · T→C · G tranversions during replication by removing an oxidized form of guanine, 8-oxo-dGTP, from the nucleotide pool, thus preventing it from mispairing with template A and maintaining replication fidelity (21). The Tn*916*-flanking region of mutant 9 (13A12) has homology with *ytmP* of *B. subtilis*. The function of this gene is yet to be determined.

Nine of the biofilm-defective mutants isolated have genes homologous to those in databases which may be important for the physiology of sessile growth. The successful identification of biofilm-associated genes indicates the utility of the polysty-

rene assay for studying bacterial biofilms. In the remaining nine biofilm-defective mutants, the DNA sequences flanking the transposon have no homology to genes in the DNA databases. This suggests that the screening performed in this study has identified previously unknown genes that are associated with biofilm formation.

The flanking regions of all the Tn*916* insertions are significantly AT rich, indicating that Tn*916* has hotspots for certain regions of the *S. gordonii* chromosome (Table 1). These AT-rich regions contained significantly more T than A. The presence of Tn*916* preferred target sites which were AT rich has also been noted in a review of conjugative transposons (57).

DISCUSSION

Kolter and Losick (37) noted that until recently, even though most microorganisms grow as biofilms and the physical structures of different biofilms are well characterized, biofilms had not been studied using molecular genetic approaches. A well-studied, genetically amenable oral bacterium, *S. gordonii* Challis, was used in this study to identify genes that are required for biofilm formation on abiotic surfaces.

The observation that *S. gordonii* Challis biofilm formation was enhanced in a minimal medium but not in a nutritionally rich environment indicates that sessile growth may represent a survival strategy in a nutritionally limited environment, as surface colonization provides advantages such as increased capture of nutrients that may be adsorbed to surfaces (64). Starvation conditions have previously been reported to initiate adhesion as bacteria attempt to exploit a source of essential nutrients which may be in short supply in the surrounding environment (38). Oral surfaces in vivo also represent a nutritionally limited environment, and biofilm formation may be required for survival, as bacteria depend on degraded salivary constituents as nutrients (40).

Interspecies variation was observed in the ability of oral streptococci to form biofilm on abiotic surfaces, which may reflect differences in the mechanisms of colonization by different streptococcal species. For example, *S. oralis* C104, which demonstrated poor biofilm-forming ability, may lack effective colonization factors for binding to abiotic surfaces but still participate in plaque formation by binding to initial colonizing cells of other species. Most human viridans streptococci participate in intrageneric coaggregation, the cell-to-cell adherence among genetically distinct streptococci, and these interactions may foster the primary colonization of the tooth surfaces (36). Furthermore, an *S. gordonii* DL1 mutant that did not coaggregate with its streptococcal partner *S. oralis* C104 exhibited wild-type levels of coaggregation with actinomyces (8).

The oral environment experiences significant fluctuations in O_2 tension, pH, and carbohydrate content due to variations in microflora, diet, and oral hygiene habits. The primary indicators of the two major dental diseases, periodontal disease and caries, are a shift in dental plaque flora from initial gram-positive facultative aerobes to mainly gram-negative anaerobes (58) and an increased acidogenicity due to bacterial metabolism of dietary sucrose (41), respectively. Some of these environmental changes, namely, in pH, osmolarity, and carbohydrate content, were found to influence streptococcal biofilm formation in vitro. As nutritional (48) and environmental (64) signals play a role in biofilm development, these observations may be useful in attempts to identify the cellular factors and molecular mechanisms involved in streptococcal biofilm formation.

Bacteria sense a large number of environmental signals and process this information into specific transcriptional responses. In gram-positive bacteria, cell-density-dependent gene expression regulatory modes appear to follow a common theme, in which the signal molecule is a posttranslationally processed peptide that is secreted by an ATP-binding cassette exporter. This peptide pheromone accumulates extracellularly in proportion to the total number of cells, providing an index of population densities (15), and functions as the input signal for the sensing component of a two-component signal transduction system (34). Therefore, these bacterial autoinduction systems represent cell-to-cell communication, which is also referred to as quorum sensing.

In *S. gordonii*, the sensing component of the two-component signal transduction system is ComD, an autophosphorylating histidine kinase. ComD is the receptor for the *comC*-encoded competence-stimulating peptide, a 50-amino-acid peptide pheromone that induces competence in the bacterial population at a critical extracellular concentration (27, 49). The second component, the cognate response regulator ComE, becomes activated after receiving the phosphoryl group from ComD at an aspartate residue and binds to specific promoter regions of appropriate target genes, therefore acting as a transcriptional factor (45).

One of the biofilm-defective mutants isolated had a transposon insertion within *comD* of *S. gordonii*, which encodes ComD (42). To our knowledge, this is the first report that cell-to-cell signaling is involved in the biofilm formation of a gram-positive species on an abiotic surface and is consistent with a previous report that the differentiation and integrity of *P. aeruginosa* biofilms are controlled by a specific quorum-sensing signal (12). A *P. aeruginosa* mutant defective in the production of *N*-(3-oxododecanoyl)-L-homoserine lactone, one of the acylhomoserine lactones that mediate quorum sensing, produced abnormal biofilms that were sensitive to the detergent biocide sodium dodecyl sulfate, indicating that a quorum-sensing signal is involved in biofilm differentiation and integrity (12). A recent study implicated cell density signaling in activation of the recovery process of nitrogen-starved *Nitrosomonas europaea* biofilms (3). Results from this study demonstrates that cell-to-cell signaling in biofilm formation may not be a characteristic restricted to *P. aeruginosa* or gram-negative bacteria.

PBPs are responsible for the assembly, maintenance, and regulation of peptidoglycan peptide structures. Identification of biofilm-associated genes that are involved in peptidoglycan biosynthesis indicates the importance of cell envelope integrity to the biofilm phenotype in streptococci, as mutations in peptidoglycan biosynthesis genes may result in cells with morphologies that lack a rigid cell envelope component.

Disruptions in genes regulating peptidoglycan synthesis are also likely to affect their ability to respond to environmental changes such as extracellular osmolarity, which is important during sessile growth. Change in environmental osmolarity can elicit structural alterations by cellular remodeling. Cellular remodeling has been shown to accompany long-term osmoadaptation, whereas the P-type ATPases are involved in acute-phase osmoadaptation (65). Identification of biofilm-associated genes that are involved in peptidoglycan biosynthesis suggest that osmoadaptation systems may play a role in biofilm formation.

One of the 18 biofilm-defective mutants (9F8) had two transposon insertions, one of which is the 5′ region of a salivary α-amylase-binding gene of *S. gordonii* (51). This is the only adhesion-related gene of *S. gordonii* found to have a potential role in biofilm formation. A previous study has shown that the mannose-sensitive hemagglutination pilus of *Vibrio cholerae*,

which is involved in biofilm formation on abiotic surfaces, is important for attachment but not pathogenicity (63). The type IV pili of *Pseudomonas aeruginosa*, which is required for biofilm formation on an abiotic surface, is also important for bacterial adhesion to eukaryotic cell surfaces and pathogenesis (47). These observations suggest that there may be an overlap in the factors required for biofilm formation and those for bacterial adhesion and/or pathogenesis in vivo. As the most abundant enzyme in human saliva, α-amylase, binds with high affinity to oral streptococci (14, 54), one of the multiple amylase-binding proteins of *S. gordonii* Challis (51) may promote biofilm formation during early plaque formation on nutritionally poor, saliva-coated tooth surfaces. This potential role of α-amylase-binding protein in biofilm formation can be determined only when the two transpositions in the biofilm-defective mutant are separated and analyzed for biofilm formation.

Although flagella and/or motility are important for biofilm formation in motile bacteria, nonmotile bacteria can also form biofilms, indicating that other genes may be involved (47, 48, 50). The successful isolation of biofilm-defective mutants clearly demonstrates the utility of the assay used. In addition, these mutants were used to identify genes that may be important in biofilm formation after initial adhesion. Nine of the 18 biofilm-defective mutants have disruptions in genetic loci that have no homology to genes in the databases. This polystyrene assay coupled with other assays such as flow cell and animal colonization studies will identify functions for some of these genes.

The relative proportion of the biofilm-deficient mutants isolated in this study (0.07%) was similar to the 0.08% obtained with Tn*917* mutants of *Staphylococcus epidermidis* were screened for biofilm deficiency (28) but lower than the 0.3% obtained from Tn*5*-based mutants of *P. aeruginosa* (48). Results from this study, together with those of a previous report (57), indicate that Tn*916* appears to preferentially transpose into AT-rich regions of the bacterial chromosome. Therefore, the biofilm-associated genes identified in this study may not represent the full complement of the genes necessary for the sessile growth of *S. gordonii*. Additional genes may be identified by insertion-duplication analysis or by using transposons from other gram-positive bacteria for mutagenesis.

In order to understand the processes involved in dental-plaque formation, different approaches, such as confocal scanning laser microscopy, which enables the study of biofilm communities without disturbance (9), and genetic approaches, need to be utilized. Two-component signal transduction systems (2) and other cell-to-cell signaling systems (18) have already become novel targets in the design of new types of microbial anti-infective therapy. Putative biofilm-associated genes may identify other processes important in sessile growth and facilitate the development of therapeutic agents that target the biofilm phenotype and cell-to-cell signaling agents and subsequently prevent the formation or promote the detachment of biofilms. Biofilm-associated genes will provide insight into the unique process of biofilm formation and may facilitate the development of therapeutic agents and strategies to control biofilm-mediated infections.

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REFERENCES

1. **Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman.** 1990. Basic local alignment search tool. J. Mol. Biol. 215:403–410.
2. **Barrett, J. F., and J. A. Hoch.** 1998. Two-component signal transduction as a target for microbial anti-infective therapy. Antimicrob. Agents Chemother. 42:1529–1536.
3. **Batchelor, S. E., M. Cooper, S. R. Chhabra, L. A. Glover, G. S. Stewart, P. Williams, and J. I. Prosser.** 1997. Cell density-regulated recovery of starved biofilm populations of ammonia-oxidizing bacteria. Appl. Environ. Microbiol. 63:2281–2286.
4. **Bloomquist, C. G., B. E. Reilly, and W. F. Liljemark.** 1996. Adherence, accumulation, and cell division of a natural adherent bacterial population. J. Bacteriol. 178:1172–1177.
5. **Cain, B. D., P. J. Norton, W. Eubanks, H. S. Nick, and C. M. Allen.** 1993. Amplification of the *bacA* gene confers bacitracin resistance to *Escherichia coli*. J. Bacteriol. 175:3784–3789.
6. **Carlsson, J.** 1972. Nutritional requirements of *Streptococcus sanguis*. Arch. Oral Biol. 17:1327–1332.
7. **Clemans, D. L., and P. E. Kolenbrander.** 1995. Identification of a 100-kilodalton putative coaggregation-mediating adhesin of *Streptococcus gordonii* DL1 (Challis). Infect. Immun. 63:4890–4893.
8. **Clemans, D. L., and P. E. Kolenbrander.** 1995. Isolation and characterization of coaggregation-defective (Cog^-) mutants of *Streptococcus gordonii* DL1 (Challis). J. Ind. Microbiol. 15:193–197.
9. **Cook, G. S., J. W. Costerton, and R. J. Lamont.** 1998. Biofilm formation by *Porphyromonas gingivalis* and *Streptococcus gordonii*. J. Periodontal Res. 33: 323–327.
10. **Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott.** 1995. Microbial biofilms. Annu. Rev. Microbiol. 49:711–745.
11. **Costerton, J. W., P. S. Stewart, and E. P. Greenberg.** 1999. Bacterial biofilms: a common cause of persistent infections. Science 284:1318–1322.
12. **Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg.** 1998. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science 280:295–298.
13. **Demuth, D. R., Y. Duan, W. Brooks, A. R. Holmes, R. McNab, and H. F. Jenkinson.** 1996. Tandem genes encode cell-surface polypeptides SspA and SspB which mediate adhesion of the oral bacterium *Streptococcus gordonii* to human and bacterial receptors. Mol. Microbiol. 20:403–413.
14. **Douglas, C. W.** 1983. The binding of human salivary alpha-amylase by oral strains of streptococcal bacteria. Arch. Oral Biol. 28:567–573.
15. **Dunny, G. M., and B. A. Leonard.** 1997. Cell-cell communication in Gram-positive bacteria. Annu. Rev. Microbiol. 51:527–564.
16. **Durack, D. T.** 1995. Prevention of infective endocarditis. N. Engl. J. Med. 332:38–44.
17. **Fenno, J. C., D. J. LeBlanc, and P. Fives-Taylor.** 1989. Nucleotide sequence analysis of a type 1 fimbrial gene of *Streptococcus sanguis* FW213. Infect. Immun. 57:3527–3533.
18. **Finch, R. G., D. I. Pritchard, B. W. Bycroft, P. Williams, and G. S. Stewart.** 1998. Quorum sensing: a novel target for anti-infective therapy. J. Antimicrob. Chemother. 42:569–571.
19. **Flannagan, S. E., L. A. Zitzow, Y. A. Su, and D. B. Clewell.** 1994. Nucleotide sequence of the 18-kb conjugative transposon Tn*916* from *Enterococcus faecalis*. Plasmid 32:350–354.
20. **Fletcher, M.** 1977. The effects of culture concentration and age, time, and temperature on bacterial attachment to polystyrene. Can. J. Microbiol. 23: 1–6.
21. **Fowler, R. G., and R. M. Schaaper.** 1997. The role of the *mutT* gene of *Escherichia coli* in maintaining replication fidelity. FEMS Microbiol. Rev. 21:43–54.
22. **Ganeshkumar, N., M. Song, and B. C. McBride.** 1988. Cloning of a *Streptococcus sanguis* adhesin which mediates binding to saliva-coated hydroxyapatite. Infect. Immun. 56:1150–1157.
23. **Gawron-Burke, C., and D. B. Clewell.** 1984. Regeneration of insertionally inactivated streptococcal DNA fragments after excision of transposon Tn*916* in *Escherichia coli*: strategy for targeting and cloning of genes from gram-positive bacteria. J. Bacteriol. 159:214–221.
24. **Gibbons, R. J.** 1989. Bacterial adhesion to oral tissues: a model for infectious diseases. J. Dent. Res. 68:750–760.
25. **Gong, K., and M. C. Herzberg.** 1997. *Streptococcus sanguis* expresses a 150-kilodalton two-domain adhesin: characterization of several independent adhesin epitopes. Infect. Immun. 65:3815–3821.
26. **Gonzalez-Barca, E., A. Fernandez-Sevilla, J. Carratala, A. Granena, and F. Gudiol.** 1996. Prospective study of 288 episodes of bacteremia in neutropenic cancer patients in a single institution. Eur. J. Clin. Microbiol. Infect. Dis. 15:291–296.
27. **Havarstein, L. S., P. Gaustad, I. F. Nes, and D. A. Morrison.** 1996. Identification of the streptococcal competence-pheromone receptor. Mol. Microbiol. 21:863–869.
28. **Heilmann, C., C. Gerke, F. Perdreau-Remington, and F. Gotz.** 1996. Characterization of Tn*917* insertion mutants of *Staphylococcus epidermidis* affected in biofilm formation. Infect. Immun. 64:277–282.
29. **Hoyle, B. D., and J. W. Costerton.** 1991. Bacterial resistance to antibiotics: the role of biofilms. Prog. Drug. Res. 37:91–105.
30. **Jenkinson, H. F.** 1986. Cell-surface proteins of *Streptococcus sanguis* associ-

ated with cell hydrophobicity and coaggregation properties. J. Gen. Microbiol. 132:1575–1589.
31. **Jenkinson, H. F.** 1994. Cell surface protein receptors in oral streptococci. FEMS Microbiol. Lett. 121:133–140.
32. **Kanamaru, K., S. Kashiwagi, and T. Mizuno.** 1993. The cyanobacterium, *Synechococcus* sp. PCC7942, possesses two distinct genes encoding cation-transporting P-type ATPases. FEBS Lett. 330:99–104.
33. **Kern, W., E. Kurrle, and T. Schmeiser.** 1990. Streptococcal bacteremia in adult patients with leukemia undergoing aggressive chemotherapy. A review of 55 cases. Infection 18:138–145.
34. **Kleerebezem, M., L. E. Quadri, O. P. Kuipers, and W. M. de Vos.** 1997. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol. Microbiol. 24:895–904.
35. **Koide, A., and J. A. Hoch.** 1994. Identification of a second oligopeptide transport system in *Bacillus subtilis* and determination of its role in sporulation. Mol. Microbiol. 13:417–426.
36. **Kolenbrander, P. E., R. N. Andersen, and L. V. Moore.** 1990. Intrageneric coaggregation among strains of human oral bacteria: potential role in primary colonization of the tooth surface. Appl. Environ. Microbiol. 56:3890–3894.
37. **Kolter, R., and R. Losick.** 1998. One for all and all for one. Science 280: 226–227.
38. **Korber, D. R., J. R. Lawrence, H. M. Lappin-Scott, and J. W. Costerton.** 1995. Growth of microorganisms on surfaces, p. 15–45. *In* H. M. Lappin-Scott and J. W. Costerton (ed.), Microbial biofilms. Cambridge University Press, Cambridge, United Kingdom.
39. **LeBlanc, D. J., and F. P. Hassell.** 1976. Transformation of *Streptococcus sanguis* Challis by plasmid deoxyribonucleic acid from *Streptococcus faecalis*. J. Bacteriol. 128:347–355.
40. **Liljemark, W. F., C. G. Bloomquist, B. E. Reilly, C. J. Bernards, D. W. Townsend, A. T. Pennock, and J. L. LeMoine.** 1997. Growth dynamics in a natural biofilm and its impact on oral disease management. Adv. Dent. Res. 11:14–23.
41. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol. Rev. 50:353–380.
42. **Lunsford, R. D., and J. London.** 1996. Natural genetic transformation in *Streptococcus gordonii*: *comX* imparts spontaneous competence on strain Wicky. J. Bacteriol. 178:5831–5835.
43. **Mack, D., M. Nedelmann, A. Krokotsch, A. Schwarzkopf, J. Heesemann, and R. Laufs.** 1994. Characterization of transposon mutants of biofilm-producing *Staphylococcus epidermidis* impaired in the accumulative phase of biofilm production: genetic identification of a hexosamine-containing polysaccharide intercellular adhesin. Infect. Immun. 62:3244–3253.
44. **Mengin-Lecreulx, D., and J. van Heijenoort.** 1996. Characterization of the essential gene *glmM* encoding phosphoglucosamine mutase in *Escherichia coli*. J. Biol. Chem. 271:32–39.
45. **Morrison, D. A.** 1997. Streptococcal competence for genetic transformation: regulation by peptide pheromones. Microb. Drug Resist. 3:27–37.
46. **Nyvad, B., and M. Kilian.** 1990. Comparison of the initial streptococcal microflora on dental enamel in caries-active and in caries-inactive individuals. Caries Res. 24:267–272.
47. **O'Toole, G. A., and R. Kolter.** 1998. Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development. Mol. Microbiol. 30:295–304.
48. **O'Toole, G. A., and R. Kolter.** 1998. Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signaling pathways: a genetic analysis. Mol. Microbiol. 28:449–461.
49. **Pestova, E. V., L. S. Havarstein, and D. A. Morrison.** 1996. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. Mol. Microbiol. 21:853–862.
50. **Pratt, L. A., and R. Kolter.** 1998. Genetic analysis of *Escherichia coli* biofilm formation: roles of flagella, motility, chemotaxis and type I pili. Mol. Microbiol. 30:285–293.
51. **Rogers, J. D., E. M. Haase, A. E. Brown, C. W. Douglas, J. P. Gwynn, and F. A. Scannapieco.** 1998. Identification and analysis of a gene (*abpA*) encoding a major amylase-binding protein in *Streptococcus gordonii*. Microbiology 144:1223–1233.
52. **Rosan, B., C. T. Baker, G. M. Nelson, R. Berman, R. J. Lamont, and D. R. Demuth.** 1989. Cloning and expression of an adhesin antigen of *Streptococcus sanguis* G9B in *Escherichia coli*. J. Gen. Microbiol. 135:531–538.
53. **Scannapieco, F. A.** 1994. Saliva-bacterium interactions in oral microbial ecology. Crit. Rev. Oral Biol. Med. 5:203–248.
54. **Scannapieco, F. A., E. J. Bergey, M. S. Reddy, and M. J. Levine.** 1989. Characterization of salivary alpha-amylase binding to *Streptococcus sanguis*. Infect. Immun. 57:2853–2863.
55. **Schilling, K. M., and W. H. Bowen.** 1992. Glucans synthesized in situ in experimental salivary pellicle function as specific binding sites for *Streptococcus mutans*. Infect. Immun. 60:284–295.
56. **Schumacher-Perdreau, F., C. Heilmann, G. Peters, F. Gotz, and G. Pulverer.** 1994. Comparative analysis of a biofilm-forming *Staphylococcus epidermidis* strain and its adhesion-positive, accumulation-negative mutant M7. FEMS Microbiol. Lett. 117:71–78.
57. **Scott, J. R., and G. G. Churchward.** 1995. Conjugative transposition. Annu. Rev. Microbiol. 49:367–397.
58. **Socransky, S. S., A. C. R. Tanner, A. D. Haffajee, J. D. Hillman, and J. M. Goodson.** 1982. Present status of studies on microbial etiology of periodontal diseases, p. 1–12. *In* R. J. Genco and S. E. Mergenhagen (ed.), Host-parasite interactions in periodontal disease. American Society for Microbiology, Washington, D.C.
59. **Switalski, L. M., H. Murchison, R. Timpl, R. Curtiss, III, and M. Hook.** 1987. Binding of laminin to oral and endocarditis strains of viridans streptococci. J. Bacteriol. 169:1095–1101.
60. **Tart, R. C., and I. van de Rijn.** 1991. Analysis of adherence of *Streptococcus defectivus* and endocarditis-associated streptococci to extracellular matrix. Infect. Immun. 59:857–862.
61. **van der Meer, J. T., J. Thompson, H. A. Valkenburg, and M. F. Michel.** 1992. Epidemiology of bacterial endocarditis in The Netherlands. I. Patient characteristics. Arch. Intern. Med. 152:1863–1868.
62. **Vickerman, M. M., D. B. Clewell, and G. W. Jones.** 1991. Sucrose-promoted accumulation of growing glucosyltransferase variants of *Streptococcus gordonii* on hydroxyapatite surfaces. Infect. Immun. 59:3523–3530.
63. **Watnick, P. I., K. J. Fullner, and R. Kolter.** 1999. A role for the mannose-sensitive hemagglutinin in biofilm formation by *Vibrio cholerae* El Tor. J. Bacteriol. 181:3606–3609.
64. **Wimpenny, J. W. T., and R. A. Colasanti.** 1997. A unifying hypothesis for the structure of microbial films based on cellular automation models. FEMS Microbiol. Ecol. 22:1–16.
65. **Wood, J. M.** 1999. Osmosensing by bacteria: signals and membrane-based sensors. Microbiol. Mol. Biol. Rev. 63:230–262.